

# Calcium mobilization in human platelets by receptor agonists and calcium-ATPase inhibitors

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Inhibitors of the endoplasmic reticulum  $\text{Ca}^{2+}$ -ATPase like thapsigargin (TG) and 2,5-di(*tert*-butyl)-1,4-benzohydroquinone (*t*BuBHQ) cause increases in cytosolic calcium in intact human platelets resulting from prevention of reuptake. A maximal concentration of TG ( $0.2 \mu\text{M}$ ) mobilized 100% of sequestered  $\text{Ca}^{2+}$  compared to the action of a receptor agonist like thrombin ( $0.1 \text{ U/ml}$ ). A maximal dose of *t*BuBHQ ( $50 \mu\text{M}$ ) stimulated release of about 40% of intracellular calcium compared to thrombin and TG. The reduced ability of *t*BuBHQ to release calcium can be explained with an inhibitory effect on the cyclooxygenase pathway ( $K_i \approx 7 \mu\text{M}$ ). Therefore *t*BuBHQ is not able to cause platelet aggregation compared to TG. In the presence of a cyclooxygenase inhibitor or a thromboxane  $\text{A}_2$  receptor antagonist the action of TG is identical to that observed with *t*BuBHQ. Generally, inhibition of calcium sequestration does not automatically result in platelet activation. In contrast to a receptor mediated activation  $\text{Ca}^{2+}$ -ATPase inhibitors require the self-amplification mechanism of endogenously formed thromboxane  $\text{A}_2$  to cause a similar response.

We conclude that the calcium store sensitive to  $\text{Ca}^{2+}$ -ATPase inhibitors is a subset of the receptor sensitive calcium pool.

Human platelet; Calcium pool; Thapsigargin; 2,5-Di(*tert*-butyl)-1,4-benzohydroquinone

## 1. INTRODUCTION

Human platelets are activated by several different agonists acting on specific receptors [1]. Included in the list of different receptor agonists thrombin and thromboxane  $\text{A}_2$  can be found. Thromboxane  $\text{A}_2$  is a potent platelet activator which serves to amplify the response to weak agonists. Thromboxane is generated by the cyclooxygenase metabolism of arachidonic acid, itself released from phospholipids upon cell activation [2]. Thrombin, thromboxane  $\text{A}_2$  and the thromboxane mimetic U46619 [3] induce platelet shape change, secretion and aggregation. During receptor occupation phospholipase C mediates the hydrolysis of phosphatidylinositol 4,5-bisphosphate [4] to yield inositol 1,4,5-trisphosphate [5,6] and 1,2-diacylglycerol [7]. The release of calcium from intracellular stores by the action of inositol 1,4,5-trisphosphate has been demonstrated [8] as well as the central role of calcium for the activation of human platelets [9,10].

Inhibitors of endoplasmic reticulum  $\text{Ca}^{2+}$ -ATPases are proving to be useful for studying intracellular calcium release and storage. Thapsigargin (TG) [11,12] and 2,5-di(*tert*-butyl)-1,4-benzohydroquinone

(*t*BuBHQ) [13,14] have been found to inhibit calcium uptake into inositol 1,4,5-trisphosphate-releasable pools by inhibition of the endoplasmic reticulum  $\text{Ca}^{2+}$ -ATPase without affecting plasma membrane  $\text{Ca}^{2+}$  transport. Therefore, both compounds cause increase in  $[\text{Ca}^{2+}]_i$  in intact cells because they release  $\text{Ca}^{2+}$  from intracellular stores resulting from prevention of reuptake.

The results reported here demonstrate that TG and *t*BuBHQ are affecting platelet aggregation differently. Although both compounds release calcium, only TG causes a full platelet activation. In contrast to TG, *t*BuBHQ has some inhibitory side effects on platelet cyclooxygenase. However, the results also indicate that the agonist induced calcium release is quantitatively different from the effect of TG and *t*BuBHQ. Therefore, we must conclude that at least two different intracellular calcium pools exist in human platelets.

## 2. MATERIALS AND METHODS

### 2.1. Materials

U46619 was purchased from Paesel, Frankfurt, Germany, thrombin was bought from Hoffmann-La Roche, Basel, Switzerland and arachidonic acid was delivered by Larodan, Malmö, Sweden. Radiochemicals were obtained from Du Pont, Dreieich, Germany. Prostacyclin and BW 755C were purchased from Sigma, Deisenhofen, Germany. Thapsigargin was ordered from Gibco, Eggenstein, Germany; *t*BuBHQ was delivered by Calbiochem, Frankfurt, Germany and sulotroban (BM 13177) was ordered from Boehringer Mannheim, Mannheim, Germany. All other materials were as previously described [15] or were obtained in the highest grade of purity available from local commercial sources.

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*Abbreviations:*  $[\text{Ca}^{2+}]_i$ , intracellular calcium; *t*BuBHQ, 2,5-di(*tert*-butyl)-1,4-benzohydroquinone; TG, thapsigargin; U46619, 15S-hydroxy-11,9-[epoxymethano]prosta-5,13-dienoic acid

### 2.2. Preparation of platelet-rich plasma and washed human platelets

Preparation of platelet-rich plasma and suspensions of washed human platelets were mainly carried out as previously outlined [15]. Briefly, citrated whole blood was centrifuged at  $200 \times g$  for 20 min and platelet-rich plasma was removed. After the addition of prostacyclin (100 ng/ml) platelets were pelleted at  $800 \times g$  (10 min), resuspended in a washing medium [15], centrifuged again and taken up in Tyrode-HEPES buffer. Aggregation was monitored with an Elvi 840 aggregometer from Logos (Milano, Italy) according to the method of Born [16]. Platelet cyclooxygenase and lipoxygenase were inhibited by preincubation with 100  $\mu$ M BW 755C for 2 min before starting each individual experiment. Platelet thromboxane receptor was blocked by preincubation with 10  $\mu$ M BM13177 for 2 min.

### 2.3. Calcium measurements

Calcium measurements in intact platelets were carried out as stated in [15]. External calcium was adjusted by the addition of 1 mM  $\text{CaCl}_2$  or by adding EGTA. Briefly, platelets were incubated with 2  $\mu$ M fura-2 AM at 37°C for 40 min, washed and resuspended in the buffer described above. Changes in fluorescence of the fura-2 loaded platelets at the excitation wavelengths of 335 and 362 nm and emission wavelengths above 450 nm were determined following the addition of antagonists and agonists. The ratio of the fluorescence is a measure of platelet cytosolic calcium [17]. Solvent carriers or inhibitors did not affect the method.

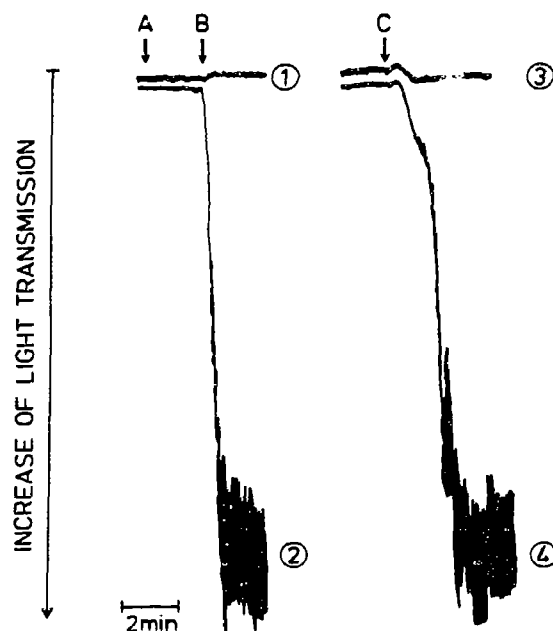
### 2.4. Arachidonic acid metabolism

Metabolism of exogenous [ $^{14}\text{C}$ ]arachidonic acid ( $1.7 \times 10^5$ – $4.5 \times 10^5$  dpm/assay) was assayed using  $3$ – $5 \times 10^5$  platelets/ $\mu$ l. Incubations were stopped after 90 s with chloroform/methanol, metabolites were extracted and spotted on silica gel 60 plates in order to separate the metabolites. Substances were localized by autoradiography and quantitated by liquid scintillation counting as outlined in [15]. Different concentrations of *t*BuBHQ were preincubated for 2 min before starting the incubation by addition of arachidonate. Results are given as mean  $\pm$  SD from 3 separate experiments.

## 3. RESULTS AND DISCUSSION

Platelet aggregation is a functional assay system to study cell activation. The experiments shown in Fig. 1 demonstrate that TG is able to induce an irreversible platelet aggregation (Fig. 1; trace 2). A concentration of 0.1  $\mu$ M TG was found to be maximally effective. Higher concentrations up to 2  $\mu$ M showed the same response. Thrombin, a commonly used platelet agonist at a concentration of 0.1 U/ml induces the same aggregatory pattern (data not shown). Therefore, our results using these two platelet agonists TG and thrombin are similar to the observation reported by Thastrup [12]. Employing *t*BuBHQ a compound with the same mechanism of action compared to TG showed a somewhat modified action.

Using low concentrations of *t*BuBHQ (up to 2.5  $\mu$ M), it sometimes was possible to induce a slow starting irreversible platelet aggregation (Fig. 1; trace 4). Normally in 9 out of 10 cases it was not possible to observe any platelet activation by *t*BuBHQ at all. Using higher concentrations of *t*BuBHQ (10  $\mu$ M; Fig. 1; trace 3) it is impossible to observe any aggregation. This is consistent for concentrations up to 100  $\mu$ M *t*BuBHQ (not shown). Inhibition of platelet cyclooxygenase by adding 100  $\mu$ M BW755C abolished the stimulatory effect of TG (Fig. 1; trace 1). Control experiments revealed that our



#### Additions:

A: BW 755C / Ethanol

B: Thapsigargin (TG)

C: 2,5-di-(*tert*-butyl)-1,4-benzohydroquinone (*t*BuBHQ)

#### Traces:

① BW 755C (100  $\mu$ M) + TG (0.1  $\mu$ M)

② TG (0.1  $\mu$ M)

③ *t*BuBHQ (10  $\mu$ M)

④ *t*BuBHQ (2.5  $\mu$ M)

Fig. 1. Thapsigargin and 2,5-di(*tert*-butyl)-1,4-benzohydroquinone affect platelet aggregation. Aggregation of washed human platelets was carried out as described in section 2. Concentrations of the test compounds and all additions are as given in the figure. Traces are representative for at least 5 similar experiments.

platelets indeed showed an aggregatory response towards arachidonic acid which was inhibited by BW755C. This allows us to determine the activity of platelet cyclooxygenase.

Further experiments trying to induce platelet aggregation with 1  $\mu$ M arachidonic acid in the presence of 10  $\mu$ M *t*BuBHQ were negative (data not shown).

In order to reveal the difference between TG and *t*BuBHQ concerning platelet activation and to explore the inhibitory effect of *t*BuBHQ on arachidonic acid induced aggregation we studied platelet eicosanoid metabolism. Washed platelets were incubated with [ $^{14}\text{C}$ ]arachidonate in the presence of increasing concentration of *t*BuBHQ as described in section 2 and as shown in Fig. 2.

In these experiments no aggregatory response was observed in any of the samples, except for the controls without *t*BuBHQ. We found a decline in cyclooxygenase pathway activity ( $\text{TxB}_2$  and HHT) and a com-

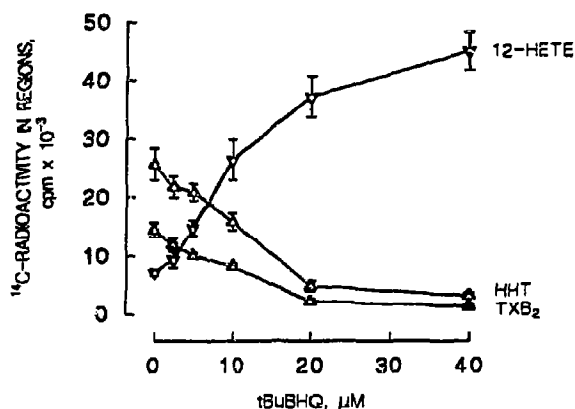


Fig. 2. Effect of *t*BuBHQ on the metabolism of [1-<sup>14</sup>C]arachidonic acid by platelets. Suspensions of washed human platelets were incubated with the indicated concentrations of *t*BuBHQ and the metabolism of [1-<sup>14</sup>C]arachidonate was studied as explained in section 2. Results are expressed as cpm corresponding to each individual metabolite: 12-HETE, 12-hydroxy-5,8,10,14-eicosatetraenoic acid; HHT, 10-hydroxyheptadecatrienoic acid; TXB<sub>2</sub>, thromboxane B<sub>2</sub>.

pensatory rise in 12-lipoxygenase activity (12-HETE). Half maximal inhibitory effects of *t*BuBHQ on platelet cyclooxygenase occurred around 7–10 μM. In contrast to *t*BuBHQ, TG does not inhibit the metabolism of arachidonic acid. Therefore inhibition of platelet cyclooxygenase by *t*BuBHQ prevents the self-amplification mechanism known to be relevant for activation of thrombocytes [18]. This observation gives a likely explanation that *t*BuBHQ does not induce platelet aggregation and also inhibits the response towards arachidonic acid. A similar situation emerges from the experiment seen in Fig. 1 (trace 1). If the transformation of arachidonic acid is inhibited, e.g. by BW755C, TG is also no longer able to aggregate platelets. It is demonstrated by using BW755C-treated thrombocytes, mimicking a situation where the self-amplification through endogenously formed thromboxane A<sub>2</sub> is not allowed that both compounds TG and *t*BuBHQ are no longer able to cause a full activation. The same situation was seen when we blocked the thromboxane A<sub>2</sub> receptor with BM13177 instead of preventing cyclooxygenase activity with BW755C, although the response towards thrombin is not influenced by either BW755C or BM13177. Therefore we conclude that the formation and action of endogenously formed thromboxane A<sub>2</sub> is absolutely required to cause a full activation by either TG or *t*BuBHQ. The inhibitory effect of *t*BuBHQ on platelet cyclooxygenase thus explains why we do not observe an aggregatory response.

We were now interested to correlate these observations with the property of the compounds to release calcium from intracellular stores. Experiments presented in Table I were carried out in the presence of 0.5 mM extracellular calcium. Therefore a cytosolic

calcium increase should represent a release from the dense tubular system, which comprises the IP<sub>3</sub>-releasable calcium store in human platelets.

Addition of thrombin causes a rapid and transient elevation of the intracellular calcium from a resting level around 50 nM to a peak maximum of nearly 200 nM cytosolic calcium. The peak calcium is referred to as 100% effect in each of the experiments. A maximal effective concentration of TG shows the same calcium release property compared to thrombin. Interestingly, *t*BuBHQ releases only roughly 30% compared to TG or thrombin. Higher concentrations of the benzohydroquinone did not further increase the response. Blocking the self-amplification mechanism of platelets by adding the thromboxane A<sub>2</sub> receptor antagonist BM13177 revealed that the calcium signal of thrombin is nearly unaffected. In contrast to the thrombin signal we noticed a marked decrease of the TG response in the presence of BM13177 to about 40% compared to controls without the receptor antagonist. The *t*BuBHQ response was not further influenced by BM13177. Qualitatively the same situation emerged when blocking platelet cyclooxygenase with BW755C instead of preventing thromboxane A<sub>2</sub> receptor activation. These observations reflect the situation when studying platelet aggregation. The calcium Ca<sup>2+</sup>-ATPase inhibitor *t*BuBHQ only partly releases sequestered calcium without showing an aggregatory response. This can be explained by the inhibitory activity of the hydroquinone on platelet cyclooxygenase. A similar situation is observed when the Ca<sup>2+</sup>-ATPase inhibitor TG is used in the presence of BW755C or BM13177. Therefore, in order to release the same amount of calcium from intracellular stores by a Ca<sup>2+</sup>-ATPase inhibitor compared to a receptor agonist the action of thromboxane A<sub>2</sub> is necessary. A receptor agonist like thrombin which is activating phospholipase C and releasing IP<sub>3</sub>, thereby causing efflux of stored calcium, is independent on the

Table I

Effect of thrombin, thapsigargin and 2,5-di(*tert*-butyl)-1,4-benzohydroquinone on intracellular calcium mobilization

Additions	Agonist-induced [Ca <sup>2+</sup> ] <sub>i</sub> increase (% of thrombin response)
Thrombin (0.1 U/ml)	100
TG (0.2 μM)	101 ± 3
<i>t</i> BuBHQ (50 μM)	32 ± 7
BM13177 + thrombin	91 ± 4
BM13177 + TG	44 ± 5
BM13177 + <i>t</i> BuBHQ	34 ± 6

Human platelets were loaded with fura-2 and intracellular calcium release was measured as described in section 2. The data are given as the means ± SD of 4 replicate determinations. Calcium increases are referred to the response of thrombin, set as 100% control value. The thromboxane A<sub>2</sub> receptor antagonist BM13177 (10 μM) was preincubated for 2 min before addition of any agonist at the concentration given

activity of platelet cyclooxygenase. This indicates that in the case of TG the stimulation of thromboxane  $A_2$ -synthesis is alone responsible for the full activation and calcium liberation. Generally, a  $Ca^{2+}$ -ATPase inhibitor by itself releases only part of the calcium which is released after receptor stimulation. Receptor-sensitive stores seem to be larger and/or only partially sensitive to the release property of  $Ca^{2+}$ -ATPase inhibitors. The results could also be interpreted in a way that the pool sensitive to  $Ca^{2+}$ -ATPase inhibitors is a subset of those sensitive after receptor stimulation.

In conclusion our findings demonstrate that  $Ca^{2+}$ -ATPase inhibitors like TG and *t*BuBHQ, were both found to inhibit the calcium uptake into  $IP_3$ -releasable pools and differ in their potency to cause a release from these stores. *t*BuBHQ, in contrast to TG, inhibits platelet cyclooxygenase thereby showing no platelet aggregation. Care should be taken using *t*BuBHQ if the cyclooxygenase pathway is involved in a transduction mechanism like in platelets. In order to release calcium equivalent to the action of a receptor agonist  $Ca^{2+}$ -ATPase inhibitors depend on the formation of thromboxane  $A_2$ . Differences in the property of receptor agonists and  $Ca^{2+}$ -ATPase inhibitors in releasing calcium may suggest different calcium pools in human platelets or a dramatically different sensitivity of one pool towards receptor agonists on one side and  $Ca^{2+}$ -ATPase inhibitors like TG and *t*BuBHQ on the other side. Our further interest will be to determine the characteristics of the different calcium stores in human platelets and the functional consequences related to platelet activation.

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